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Human Factor IX Binds to Specific Sites on the Collagenous Domain of Collagen IV*

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The primary region of factor IX that mediates binding to bovine aortic endothelial cells resides in residues 3–11 of the N-terminal region known as the Gla domain. Recently, it was proposed that the observed binding to endothelial cells is actually a measure of the interaction between factor IX and collagen IV (Cheung, W. F., van den Born, J., Kuhn, K., Kjellen, L., Hudson, B. G., and Stafford, D. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11068–11073). To confirm that factor IX binds to collagen IV and to examine the specificity of this interaction, we used scanning force microscopy to examine factor IX binding to collagen IV. We imaged collagen IV in the presence and the absence of factor IX and observed specific interactions between factor IX and collagen IV. Our results demonstrate that factor IX binds to collagen IV at specific sites in the collagenous domain ~98 and ~50 nm from the C-terminal pepsin-cleaved end.

Factor IX (F.IX)[1] is a 57-kDa zymogen of a serine protease that participates in blood coagulation. Activated F.IX (factor IXa), with its nonenzymatic cofactor, factor VIIIa, activates factor X to factor Xa, which converts prothrombin to thrombin in the penultimate step of the coagulation cascade. Mutations in F.IX result in the bleeding disorder hemophilia B (1).

It has been observed that F.IX binds to bovine aortic endothelial cells with high affinity (5 nM K_d) (2, 3) and that mutating residues 3–11 of F.IX eliminates this binding (4). Recently, Cheung *et al*. (5) observed that in the presence of heparin, F.IX has a similar binding affinity to collagen IV (C.IV) and suggested that the endothelial cell binding site is C.IV.

C.IV is an extracellular matrix protein and a major component of the basement membrane region of endothelial cells. C.IV is a right-handed triple helix composed of two α1 chains and one α2 chain, each of which is a left-handed helix (6). Each of these chains encodes the repeating G*XX* sequence (where the first *X* and second *X* can be any amino acid, but are frequently proline and hydroxyproline, respectively) characteristic of collagenous proteins (7–11). Two noncollagenous regions flank the triple helical collagenous domain: a 7S domain on the N-terminal end and an NC1 domain on the C-terminal end. The 7S domain mediates tetramer formation between four C.IV monomers joined at their N-terminal ends, whereas the globular NC1 domain participates in dimer formation between the C-terminal ends of two C.IV monomers (12, 13). The NC1 and 7S domains mediate polymerization to form an extensive C.IV network. Treatment of this network with pepsin produces C.IV tetramers linked by their 7S domains (see Fig. 2). These tetramers can be used in *in vitro* studies (12, 14).

Denaturation of C.IV with dithiothreitol or heat eliminates binding (5), suggesting that F.IX binding to C.IV requires the intact triple helix. It is not known, however, whether F.IX binds to a single specific site or if there are numerous F.IX recognition sites on the C.IV molecule. We used scanning force microscopy (SFM) to localize F.IX binding on C.IV by directly visualizing the interaction between F.IX and C.IV.

SFM (also called atomic force microscopy) is a powerful tool for investigating the structure of protein-protein and protein-nucleic acid complexes. SFM produces a topographic image of a sample by scanning a surface with a nanometer scale tip attached to a flexible cantilever. Consequently, it is possible to obtain three-dimensional images of macromolecules that are deposited on very smooth surfaces such as mica (reviewed in Refs. 15 and 16). Because of the sensitivity of the instrument to variations in the dimensions of macromolecules, SFM is an excellent method for investigating the binding of F.IX to C.IV. In this study, we imaged both F.IX and C.IV separately and F.IX bound to C.IV tetramers. We observed two specific F.IX binding sites and identified F.IX that was nonspecifically bound to C.IV.

EXPERIMENTAL PROCEDURES

Proteins—F.IX was purchased from Enzyme Research Laboratories (South Bend, IN). Human C.IV tetramers (from placenta) and heparin were purchased from Sigma. Antibody A-5, which recognizes the F.IX catalytic domain (17), was a generous gift from Dr. Kenneth Smith of Emory University.

Solid Phase Binding Assay—To assure that the material used in this study was functionally identical to that used by Cheung *et al*. (5), we performed the binding assay as described (5) and obtained a K_d of ~60 nM, similar to that obtained in the earlier study.

Sample Preparation—Antibody A-5, which does not affect C.IV binding,[2] was used to increase the apparent size of the F.IX molecule to make F.IX more easily identifiable. To form F.IX and A-5 (F.IX·A-5) complexes, F.IX (90 nM) and A-5 (18 nM) were incubated in TBS-CM (20 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 8.0) for 2 h at 4 °C. To form complexes between C.IV and F.IX·A-5, C.IV (50 μg/ml) was incubated with F.IX (90 nM) and A-5 (90 nM) for 2 h at 4 °C in the presence of 1 μg/ml heparin in TBS-CM (5). As a control, A-5 (18 nM) was incubated with C.IV (50 μg/ml) and heparin (1 μg/ml) in the absence of F.IX. Each sample was diluted 1:15 or 1:20 into TBS-CM, and 10 μl of the diluted sample were deposited onto a freshly cleaved mica surface. Consequently, the concentrations of F.IX and heparin at the time of deposition were ~6 nM each, which is below the K_d values for both F.IX and heparin binding to C.IV. These concentrations gave optimal coverage and visibility of individual collagen molecules. After allowing the samples to set for 5 s, the surfaces were thoroughly rinsed five times with ~0.5 ml of doubly distilled water using a wash bottle. This procedure removes salt but leaves proteins bound to the surface,

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[1] The abbreviations used are: F.IX, factor IX; C.IV, collagen IV; SFM, scanning force microscopy.

[2] W.-F. Cheung and D. W. Stafford, unpublished results.

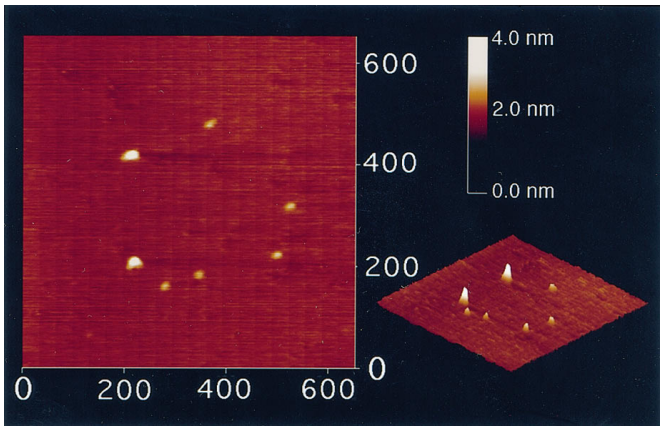


FIG. 1. **Top and surface (*inset*) views of free F.IX and F.IX·A-5 complexes.** The smaller (less intense) structures represent free F.IX, whereas the taller (more intense) structures represent F.IX·A-5 complexes. The observed increase in size of F.IX·A-5 complexes to free F.IX is expected from the increase in molecular weight of the protein upon antibody binding.

i.e. F.IX·A-5 complexes bound to C.IV remained bound to the surface. The surfaces were then dried with N_2 and imaged in air.

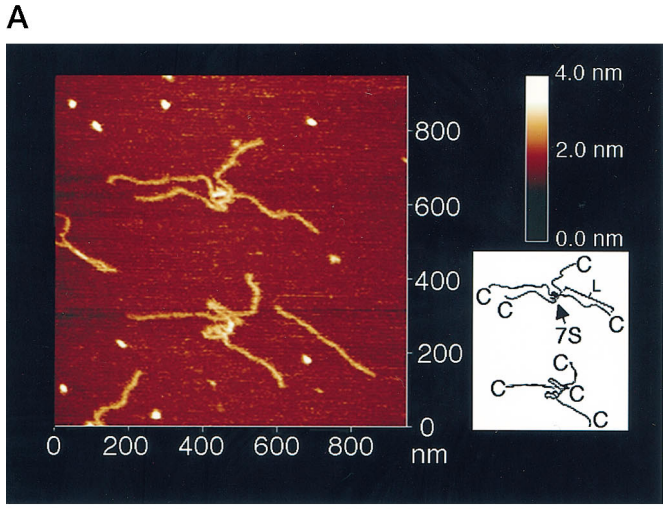
SFM-TM Hardware—Images were obtained in *tapping mode* using a Nanoscope IIIa from Digital Instruments (Santa Barbara, CA). Silicon cantilevers (125 μm) with a resonance frequency between 300 and 350 kHz were used (Digital Instruments). The scan rate was 1.85 lines/s with 512 lines/image. Images were flattened prior to analysis.

RESULTS

Visualizing Factor IX and A-5 and Collagen IV—F.IX, A-5, and C.IV molecules were stable under repeated scans and had consistent appearances in several different depositions. F.IX molecules were globular, with a diameter of 16 ± 2 nm (n = 10, where n is the number of molecules measured; Fig. 1). F.IX·A-5 complexes were globular with a diameter of 23 ± 3 nm and a height approximately twice that of F.IX (n = 10; Fig. 1). C.IV molecules were tetrameric (Fig. 2); however, some C.IV structures contained less than four monomers. The three "bumps" indicated by small arrows in the 7S domain in Fig. 3*A* were observed on tetrameric C.IV both in the presence and the absence of F.IX·A-5.

C.IV deposited in the absence of F.IX and A-5 was essentially free of features having F.IX·A-5 dimensions. In depositions with both F.IX·A-5 complexes and C.IV, features on C.IV with dimensions consistent with free F.IX·A-5 complexes were identified as bound complexes (Fig. 3). To assure that the complexes were a result of F.IX association and not an interaction between A-5 and C.IV, we imaged both A-5 and C.IV alone (n = 30) and F.IX·A-5 complexes and C.IV in the presence of 2 mM EDTA (n = 18), which inhibits F.IX binding to C.IV. In these images, molecules associated with C.IV were randomly bound to the collagenous domain (data not shown). Furthermore, in the presence of EDTA, only a few F.IX molecules were associated with C.IV (data not shown).

Data Analysis—Only collagen molecules with clearly visible N (7S domain) and C termini were analyzed. All distances were measured using Digital Instruments software. Standard error of measurement that was determined by measuring individual collagen strands was ± 13 nm. The length distribution of the collagenous domains (see legend to Fig. 2) of C.IV molecules deposited in the absence of F.IX. A-5 was gaussian, with an average of 318 nm and a standard deviation of ±48. 132 complexes were analyzed and two peaks (located at 98 ± 13 and 50 ± 13 nm) were observed (Fig. 4). These peaks were not due to differences in monomer length, because the average lengths of monomers with complexes bound at 98 and 50 nm were



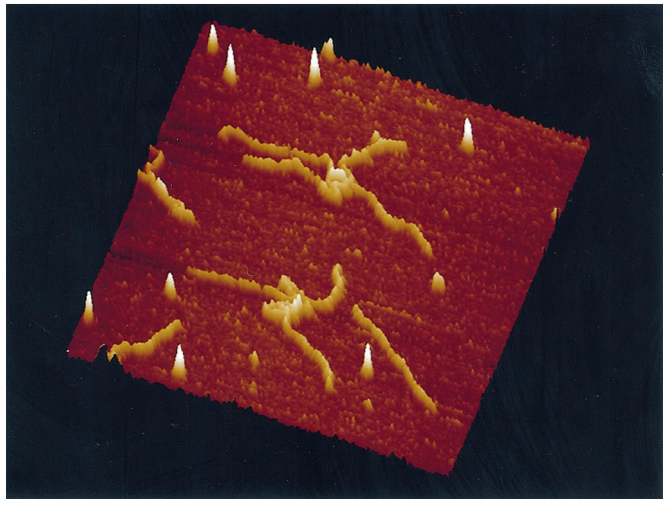


FIG. 2. **Top (*A*) and surface (*B*) views of C.IV tetramers and free F.IX·A-5 complexes.** The tetramers are from a deposition that contains F.IX and A-5. C.IV deposited in the absence of F.IX and A-5 appears identical to these images. The scales are the same in views *A* and *B*. The 7S domain and *C* termini of the molecules in the tetramer are labeled in the *inset* to *A*. A *bracket* (*L*) indicates the collagenous domain.

316 ± 24 and 315 ± 28 nm, respectively. Both binding sites were statistically significant when examined by the goodness of fit of a binomial distribution ($p < 10^{-10}$ and 0.004 for the binding sites located 98 and 50 nm from the C-terminal end, respectively).

DISCUSSION

Our results support earlier conclusions (5) that F.IX recognizes and binds with high affinity to C.IV and not to a contaminant protein in the C.IV preparation. The data in Fig. 4 reveal that F.IX binding is localized to the C-terminal portion of the C.IV monomer within two sharp peaks centered at ~98 and ~50 nm from the C-terminal end. The larger peak at 98 nm likely represents a high affinity, specific F.IX binding site on the collagenous domain, whereas the smaller peak may represent a secondary, specific F.IX binding site, having 3–4-fold lower affinity for C.IV than the primary site.

Because the breadth of the entire F.IX distribution is too large to be explained by errors in our measurements, we suggest that the distribution represents both specifically bound F.IX and F.IX that is nonspecifically bound to the C.IV molecule. The stability of the specific complex is likely to be a result

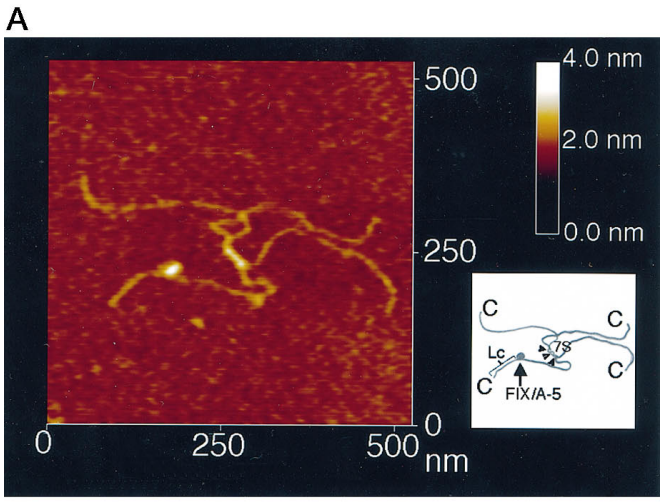


FIG. 3. **Top (*A*) and surface (*B*) views of F.IX·A-5 complexes bound to C.IV.** C termini, 7S domains, F.IX·A-5 complexes, and the region measured for Fig. 4 (*bracket*, *Lc*) are indicated in the *inset* to *A*. The scale in *B* is the same as in Fig. 1.

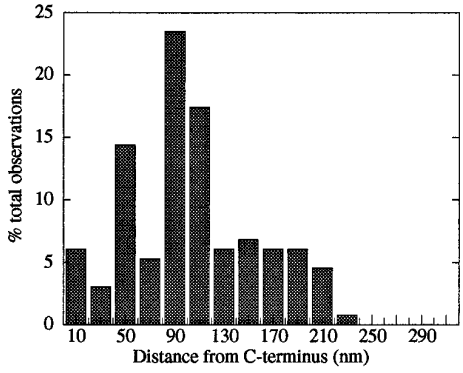


FIG. 4. **Distribution of distances of the F.IX·A-5 complexes from the free (C-terminal) end of the C.IV monomers.** 132 F.IX·A-5-bound collagen molecules were analyzed. The data were divided into 20 nm bins for graphing; however, changes in the bin size (±5 nm) did not affect the significance of the peaks. Both of the peaks were statistically significant ($p < 0.0004$).

of nonspecific interactions with the triple helix, as well as interactions with specific amino acid residues. Therefore, it is reasonable to expect F.IX to bind nonspecifically along the length of C.IV. The large number of nonspecifically bound F.IX molecules on the C.IV monomer strongly supports earlier suggestions that F.IX interacts with the triple helix (5) and not with a single peptide chain, because nonspecific binding would not be expected if F.IX were interacting with a conformationally distinct looped out region. This nonspecific binding is analogous to that observed for proteins that bind to DNA, which, like collagen, is also a long regular helical molecule. Indeed, the structurally regular "backbone" of the triple helical region may provide a significant portion of the binding free energy, as is observed for DNA binding proteins (18–20).

In general, the number of F.IX molecules bound specifically and nonspecifically to C.IV is determined by the concentration of nonspecific and specific binding sites and the relative binding affinities to these sites. According to a lattice binding model (21), if F.IX interacts with 5–30 residues of the triple helix, the number of potential binding sites for a single F.IX molecule is approximately 1300. If we assume that F.IX has a single, specific binding site, the concentration of nonspecific sites is 1300 times greater than the specific site. Consequently, if F.IX has a 1300-fold higher affinity for its specific site *versus* its nonspecific sites, we expect to observe ~50% of the F.IX molecules to be bound specifically and 50% to be bound nonspecifically. Such a difference in specific and nonspecific binding affinities is typical for DNA binding proteins (18–20). In our study, ~55% of F.IX molecules is specifically bound to the C.IV molecule, indicating that F.IX has ~1000-fold greater affinity for its specific sites than its nonspecific sites.

Surprisingly, the nonspecifically bound complexes were adjacent to the high affinity binding site (within 100 nm) and were not spread across the entire length of the molecule. This result is consistent with a mechanism of one-dimensional diffusion in which microscopic associations and dissociations between F.IX and C.IV permit F.IX to "slide" or "hop" along the C.IV molecule in transit to and from its specific site (22, 23). In the absence of this mechanism, we would expect nonspecific binding of F.IX to be governed by three-dimensional, diffusion-controlled, random protein-protein associations, and therefore, we would expect to observe F.IX binding across the entire length of the C.IV molecule. Significantly, a similar distribution of binding has been observed for Cro binding to DNA, which exhibits kinetics indicative of one-dimensional diffusion (24).[3] These results for F.IX binding to C.IV suggest that one-dimensional diffusion may be important for protein-protein interactions involving long regular structures, as well as for DNA binding proteins.

From our measurements of the position of the specific F.IX binding sites and from data on triple helix structure, we can identify the regions of amino acids that mediate specific F.IX binding to C.IV. C.IV differs from fibrillar collagens, because it contains interruptions of noncollagenous sequences within its collagenous repeats. Because these interruptions do not alter triple helix propagation in the collagenous domain, it is unlikely that they disrupt the triple-helical character of the collagenous domain (6, 25–28). Accordingly, we assumed a linear dependence of helical length for the 1304 amino acid residues in the collagenous domain. This assumption allowed us to localize F.IX binding to specific amino acid regions by measuring the position of F.IX along the helix. From the 318 nm measured length of the collagenous domain, we calculated a distance of 2.44 Å per amino acid along each chain in the collagenous domain, which is consistent with the published value for a synthetic triple helical peptide (2.9 Å) (28). Using this value, we concluded that the specific F.IX binding site located 98 ± 13 nm from the C-terminal end corresponds to residues 985–1092 of the α1 chain and residues 1030–1137 of the α2 chain (Fig. 5). Similarly, the secondary specific F.IX binding site located 50 ± 13 nm from the C-terminal end

[3] D. A. Erie, unpublished observation.

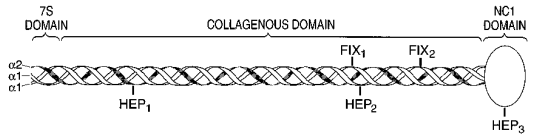


FIG. 5. **Schematic representation of a C.IV molecule.** The two α1 chains are depicted as *open ribbons*, whereas the α2 chain is shown as a *closed ribbon*. The 7S, collagenous and NC1 domains are labeled above the diagram. The approximate locations of the mapped F.IX (FIX_1 and FIX_2) and heparin (HEP_1, HEP_2, and HEP_3) (31) binding sites are indicated *above* and *below* the diagram. Our experiments were performed with pepsin-treated C.IV that does not contain the NC1 domain.

corresponds to residues 1182–1288 of the α1 chain and residues 1227–1333 of the α2 chain.

A search of the amino acids surrounding the mapped F.IX binding regions reveals several noncollagenous inserts within the G*XX* collagenous repeats. Noncollagenous sequences at residues 1056–1057 (GI) of the α1 chain and 1033 (K) and 1097–1100 (INLP) of the α2 chain are located within the identified primary specific binding region (FIX_1 in Fig. 5). Additionally, noncollagenous sequences at residues 939–947 (DKVDMGSMK) of the α1 chain and 980–990 (PVILPGMKDIK) of the α2 chain flank the identified region. Residues 1182–1288 (α1 chain), which correspond to the secondary peak at ~50 nm from the C-terminal end (FIX_2 in Fig. 5), also contain several noncollagenous sequences: residues 1231–1232 (TE) of the α1 chain, and residues 1235–1238 (NTLP), 1272–1277 (PPSNIS), and 1305–1307 (ALP) of the α2 chain. Each of these noncollagenous regions most likely maintains the triple helical nature of the collagenous domain (6, 25–28) and possibly provides considerable specificity within the molecule. These sequences, therefore, merit investigation in identifying the specific F.IX binding sites on C.IV. Because several groups have successfully constructed and characterized structurally intact triple helical peptides (26–30), it may be possible to identify specific F.IX-binding amino acid residues on C.IV using synthetic peptides or alternatively, using site-directed mutagenesis.

Because heparin has been shown to increase the binding affinity of F.IX to C.IV (5), it is interesting that the primary specific F.IX binding site was located in the same region as a previously identified heparin binding site (31) (FIX_1 and HEP_2 in Fig. 5). It is possible that heparin increases the affinity of F.IX to the primary site on C.IV by binding to both F.IX and to C.IV and stabilizing their interaction. It is unlikely, however, that heparin is solely responsible for mediating this interaction because F.IX binds to C.IV in the absence of heparin (5) and because the heparin concentration used in this study is below the measured K_d for the heparin-C.IV interaction. In addition, we did not observe F.IX binding to the heparin binding site proximal to the 7S domain (HEP_1 in Fig. 5) (31), whereas we did observe specific binding of F.IX ~50 nm from the C terminus (FIX_2 in Fig. 5) where there is no evidence for heparin binding. Because two groups previously reported that heparin fails to bind to pepsin-treated (tetrameric) C.IV (32, 33), whereas Cheung *et al*. (5) showed that heparin increases the affinity between F.IX and tetrameric C.IV, the exact role of heparin in the interaction between F.IX and C.IV remains unclear.

The collagens previously have been implicated in a variety of physiological activities. Collagen types I, II, III, and IV induce platelet adhesion (reviewed in Ref. 34). C.IV plays a role in cell migration and cell binding during tumor metastasis (35). Indeed, specific sites on C.IV have been identified that bind heparin (31) and the integrins $\alpha1\beta1$ (36), $\alpha2\beta1$ (37), and $\alpha3\beta1$ (38). Given the unique site on F.IX that interacts with C.IV and the specificity of the recognition sites on C.IV, these results strongly suggest that the F.IX-C.IV interaction is physiologically relevant. Whether this interaction is required for normal hemostasis remains to be determined.

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